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SHORT COMMUNICATION

G to A transitions and G to T transversions in codon 12 of the Ki-*ras* oncogene isolated from mouse lung tumors induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and related DNA methylating and pyridyloxobutylating agents

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Lung tumors were induced in A/J mice by the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and the related compounds acetoxymethylmethylnitrosamine (AMMN) and 4-acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc). NNK both methylates and pyridyloxobutylates DNA while AMMN and NNKOAc only methylate or pyridyloxobutylate DNA, respectively. The lung tumors were analyzed for mutations in the Ki-*ras* oncogene by PCR amplification followed by either restriction fragment length polymorphism, hybridization, or sequencing procedures. NNK induced GGT to GAT mutations in codon 12 (26 of 28 samples analyzed). AMMN induced GGT to GAT mutations in 18 of 18 samples. In contrast, NNKOAc induced a variety of changes including GGT to GAT (8/21), GGT to TGT (5/21) and GGT to GTT (4/21) mutations. These results demonstrate that DNA methylation causes mainly G to A transitions in the Ki-*ras* gene of A/J mouse lung tumors, consistent with previous results and a role for O^6-methylguanine, while DNA pyridyloxobutylation induces G to A transitions as well as G to T transversions, perhaps due to the steric bulk of the adducts which are formed. The results are discussed with respect to mutations observed in rodent and human lung tumors.

The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK*) is a potent pulmonary carcinogen inducing predominantly adenocarcinoma in mice, rats, and hamsters (1–4). The total doses of NNK required to produce lung tumors in rats are similar to the total doses to which smokers are exposed, as determined from the levels of NNK in cigarettes and the amounts of its metabolites in smokers' urine (5,6). DNA and hemoglobin adducts produced by NNK in the rat and mouse have also been detected in smokers and their levels are higher than in non-smokers (7–9). These data support the hypothesis that NNK plays a role in lung cancer etiology in smokers.

The metabolic activation of NNK proceeds by α-hydroxylation of its methylene and methyl groups as illustrated in Figure 1. Methylene hydroxylation leads to DNA methylation while methyl hydroxylation leads to DNA pyridyloxobutylation. In order to individually assess the contributions of these pathways to carcinogenesis by NNK, we have used the model compounds acetoxymethylmethylnitrosamine (AMMN) and 4-(acetoxymethylmethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc) (10). AMMN undergoes hydrolysis catalyzed by esterase to produce a methylating agent which is similar or identical to that formed by methylene hydroxylation of NNK while hydrolysis of NNKOAc produces a pyridyloxobutylating agent. Previous studies carried out in A/J mice have shown that AMMN methylates guanines in lung DNA producing O^6-methylguanine (O^6-mG) while NNKOAc pyridyloxobutylates lung DNA as determined by quantitation of HPB-releasing adducts (10). Comparisons of adduct levels and tumor induction in A/J mice by NNK, AMMN, NNKOAc, and mixtures of these compounds strongly support the hypothesis that formation of O^6-mG is a critical event in A/J mouse lung tumorigenesis by NNK (10). These studies are in agreement with the conclusions reached by Belinksy *et al.* who analyzed Ki-*ras* mutations in lung tumors induced by NNK in A/J mice (11). They found a high percentage of G-A transitions in the second base of codon 12, which could result from an O^6-mG adduct. The same mutations have been observed in hamster lung tumors induced by NNK (12).

In this study, we analyzed mutations in codon 12 of the Ki-*ras* gene in lung tumors isolated from A/J mice treated with NNK, AMMN or NNKOAc. Our purpose was two-fold: to determine whether the G to A transitions induced by NNK resulted from the DNA methylation or pyridyloxobutylation pathway and to determine the mutational consequences of DNA pyridyloxobutylation in Ki-*ras* codon 12.

Forty female A/JCr mice, 5 weeks old, were obtained from the National Cancer Institute, Frederick, MD. Two weeks later, they were divided into four groups of 10 mice each and housed 10 per cage under standard conditions. The mice were given i.p. injections of the appropriate compound in 0.1 ml of saline or of 0.1 ml saline alone. NNK and NNKOAc were synthesized (13,14). AMMN was obtained from the National Cancer Institute

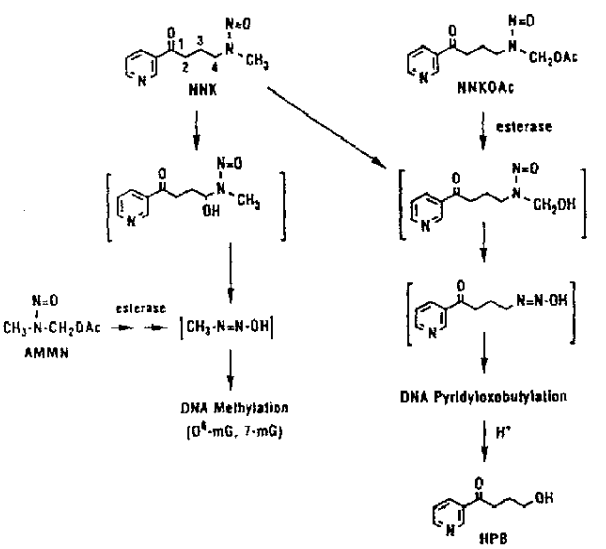

Fig. 1. Conversion of NNK, AMMN or NNKOAc to intermediates which methylate or pyridyloxobutylate DNA.

*Abbreviations: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; AMMN, acetoxymethylmethylnitrosamine, NNKOAc, 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone; O^6-mG, O^6-methylguanine; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone

Chemical Carcinogen Reference Standard Repository. Injections were given on Mondays, Wednesdays and Fridays for a total of four injections of AMMN, 17 injections of NNKOAc and 22 injections of NNK. Each injection was comprised of 5 μmol NNK, 2.5 μmol NNKOAc or 0.75 μmol AMMN. The total doses of each compound were (μmol/mg): AMMN (3/0.4); NNKOAc (42.5/11.3); NNK (110/22.8). The mice were killed 35 weeks after the first injection and lung tumors were removed and stored at −80°C. In previous studies, an essentially identical protocol for NNK resulted in 38 lung tumors per mouse, consisting of adenomas and adenocarcinomas (15).

In the group treated with NNK, there were 36 lung tumors per animal, in good agreement with the previous results (15). The groups treated with AMMN and NNKOAc had 39 and 13 lung tumors per mouse respectively. Only one tumor was observed in the vehicle control group.

To determine the frequency and type of Ki-*ras* oncogene mutations in these tumors, we used three independent procedures: PCR followed by RFLP analysis, PCR followed by hybridization with specific probes, and PCR followed by cloning and sequencing. Genomic DNA was prepared from each tumor using standard procedures (16). The tumors ranged in size from 0.5 to 3 mm and yielded on average ~15 μg DNA. Initially, we were interested in identifying the frequency of Ki-*ras* mutations caused by each compound. This was accomplished using PCR together with RFLP analysis, as previously described for the analysis of human and rat *ras* oncogene sequences (16,17). The procedure is outlined in Figure 2A. The 5′ primer was modified to create a *Bst*NI restriction enzyme site (CCTGG) when combined with a normal codon 12 Ki-*ras* allele. Thus, the 5′ primer was 5′ ACTGAGTATAAACTTGTGGTAGTTGGCCCT 3′. Changes in either the first or second base of codon 12 would result in the loss of the restriction site. The 3′ primer was also modified to create a restriction site for *Bst*NI. Therefore, the 3′ primer was 5′-GCAGCGTTACCTCTATCCTGGGGTCGT-ACT 3′. The CCTGG restriction site in this primer served as a positive control for *Bst*NI activity. One μg of genomic DNA was amplified using 15 pmol of each primer, 0.2 mM dNTP (Cetus, Norwalk, CT) and Taq polymerase (Cetus) as previously described (16). The PCR reaction was carried out using a microcycler (Ericomp, San Diego, CA) for 35 cycles consisting of 1 min at 94°C, 1 min at 53°C and 1 min at 72°C. A 20 μl aliquot of the amplified DNA was digested with *Bst*NI (New York Biolabs, Commack, NY) (5 U in buffer consisting of 50 mM NaCl, 10 mM Tris−HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.9), BSA 100 μg/ml for 1 h at 60°C) followed by separation on a native acrylamide gel (15%) and analysis via ethidium bromide staining. The results shown in Figure 2B illustrate that this procedure allows a clear distinction between normal and mutant Ki-*ras* codon 12 alleles; the latter have a characteristic 100 bp fragment.

The results of the RFLP analysis showed that all tumors from animals treated with NNK (n = 20) or AMMN (n = 14) and 15 of 19 NNKOAc derived tumors contained a mutation in codon 12 of the Ki-*ras* gene. The RFLP analysis would have detected mutations in the first two bases of codon 12.

To determine the nature of the mutations in codon 12, DNA from these 53 tumors and an additional 14 samples was PCR amplified using primers purchased from Clontech (Palo Alto, CA) and the amplified material was dot-blotted on nylon membranes (Duralon, Stratagene, LaJolla, CA). It was hybridized with ^{32}P-labelled probes (Clontech) specific for each possible mutation

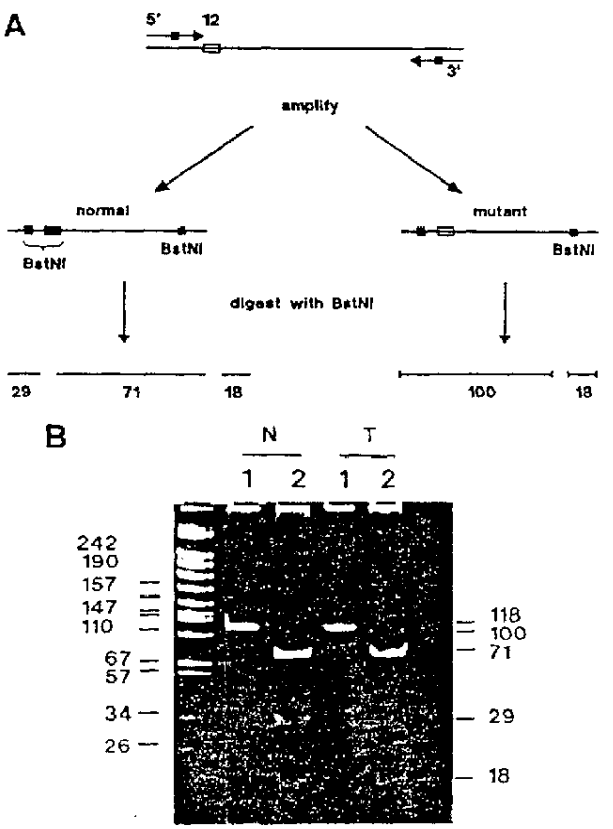

Fig. 2. A. Schematic representation of RFLP−PCR analysis designed for mouse Ki-*ras* codon 12 oncogene. Digestion of PCR-amplified products will yield three fragments that are sized 71, 29 and 18 bp from normal alleles and 100 and 18 bp from mutant alleles. A mixture of 100, 71, 29 and 18 bp fragments indicates the presence of one mutant and one normal allele. B Representative analysis by RFLP−PCR. Shown is analysis of DNA prepared from normal lung tissue (N) and from a lung tumor (T), that was amplified to generate the expected 118 bp fragment (lane 1) and then digested with *Bst*NI (lane 2). Mol. wts markers shown on the left panel are *Hpa*II (Promega Corporation, Madison, WI) digest of SKII+ plasmid (Stratagene, LaJolla, CA). Mol. wts of the fragments obtained in the RFLP−PCR analysis are shown on the right panel.

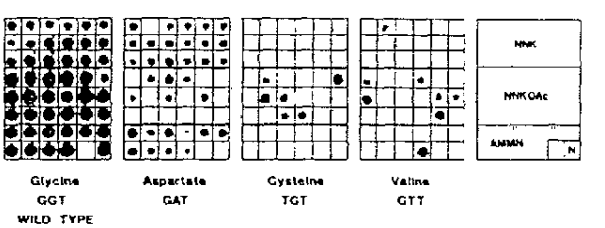

Fig. 3. Identification of base pair substitutions in codon 12 of mouse Ki-*ras* oncogene using dot blot hybridization. Genomic DNA from tumors, represented by boxes in the grid, induced by NNK, NNKOAc or AMMN as well as from normal lung (N) or no DNA (control) were amplified and dot-blotted onto nylon membranes as outlined in the right panel. DNA sequences that are specific for each of the possible point mutations within codon 12 of Ki-*ras* were labeled and independently hybridized with the dotted DNA. Following wash to remove non-specific signals, the membrane was analyzed on a computerized radioimaging blot analyzer (AMBIS, San Diego, CA). The positive signals and the respective mutation are outlined in each panel.

in codon 12 of the mouse Ki-*ras* oncogene. Representative hybridization patterns are shown in Figure 3.

This analysis demonstrated that tumors induced by NNK and AMMN contained 96 and 100% GGT to GAT mutations respectively, while tumors induced by NNKOAc contained a variety of mutations including GGT to GAT, GGT to GTT and GGT to TGT. Six of the tumors analyzed by this procedure revealed more than one mutation in addition to the wild type allele, as indicated in Figure 3. These samples were further analyzed. These DNA samples were amplified and cloned into TA Cloning™ Vector (pCR™ II) (In Vitrogen Corp., San Diego, CA). Following transformation into INVαF competent *Escherichia coli* as described by the manufacturer, several independent clones were selected and subjected to sequencing, using a Sequence 2.0 kit (USB, Cleveland, OH). The results of this analysis showed that these six samples contained only a single mutation suggesting that one of the hybridization signals may have been a false positive. However, it is also possible that the tumor sample may have been contaminated by material from a closely adjacent tumor. The composite results of the hybridization and sequencing experiments on 67 tumors are summarized in Table I.

The results agree with published data which show that a G to A transition at the second base of codon 12 is the predominant mutation found in lung tumors induced by treatment of A/J mice with NNK (11). Tumors induced by AMMN in A/J mice have not been previously analyzed for Ki-*ras* mutations but a related methylating agent, methylnitrosourea, induced exclusively G to A transitions at the second base of codon 12, as seen in this study for AMMN (18). These results are consistent with a mechanism involving methylation of the Ki-*ras* gene. One of the adducts which is formed, O^6-mG, is known to have miscoding properties which would account for the observed G to A transition (19,20). Previous studies of tumor induction in A/J mice by NNK, employing deuterated analogues as well as related model compounds such as AMMN and NNKOAc, are entirely consistent with a critical role for persistent O^6-mG in mouse lung tumorigenesis (10,21).

While NNK and AMMN induced primarily G to A transition mutations, tumors isolated from mice treated with NNKOAc contained a mixture of mutations in codon 12 of the Ki-*ras* gene; 47% were G to A transitions and 53% were G to T transversions. Our previous studies have shown that the main effect of DNA pyridyloxobutylation in A/J mouse lung tumorigenesis is to enhance the persistence of O^6-mG and to enhance tumorigenesis, most likely through the ability of pyridyloxobutylated DNA to inhibit the repair of O^6-mG (10,22). Thus, it would be expected that the spectrum of mutations induced by NNK in codon 12 of the Ki-*ras* gene isolated from mouse lung tumors should more closely resemble that induced by AMMN than by NNKOAc, which is what we observed in this study. Nevertheless, the results suggest that some of the G to A transitions induced by NNK may result from DNA pyridyloxobutylation.

Table I. Analysis of mutations in codon 12 of the Ki-*ras* gene isolated from lung tumors induced by NNK, AMMN and NNKOAc

Compound	No. of tumors	No. of tumors (%) with codon 12			
		GGT (wt)	GAT	TGT	GTT
NNK	28	0	26 (96.4)	2 (3.6)	0
NNKOAc	21	4 (19.1)	8 (38.0)	5 (23.8)	4 (19.1)
AMMN	18	0	18 (100)	0	0

The ability of NNKOAc to induce a significant percentage of G to T transversions is of interest. The structures of the DNA adducts formed by NNKOAc have not been characterized because these adducts, while relatively stable in double stranded DNA *in vitro* and in both mouse and rat lung *in vivo*, have not been isolatable upon enzymatic or chemical hydrolysis of the DNA conditions which reproducibly result in the release of HPB (23,24). Presently available data indicate that NNKOAc forms more than one HPB releasing adduct in DNA. These adducts will be sterically larger than O^6-mG and might be expected to produce G to T transversions as observed with a number of other relatively large DNA damaging agents such as electrophilic metabolites of aromatic amines and polynuclear aromatic hydrocarbons (18,20).

While activation of the Ki-*ras* gene by O^6-mG appears to play an important role in NNK induced mouse lung tumorigenesis, the relevant events are less clear in the rat lung which is also a major target for induction of adenocarcinoma by NNK. Activated Ki-*ras* genes have not been isolated from rat lung tumors induced by NNK (25). In contrast to the mouse, carcinogenicity studies of deuterium labeled analogues of NNK in the rat did not provide a clear answer regarding the role of DNA methylation or pyridyloxobutylation in lung tumorigenesis by NNK (26). Although formation of O^6-mG in Clara cells of the lung correlated with lung tumorigenesis by various doses of NNK in the rat, the tumors apparently originated from type II cells where this correlation was not observed (4). Thus, further studies are required to determine the relative roles of DNA methylation and pyridyloxobutylation in rat lung tumorigenesis by NNK.

Activated Ki-*ras* genes with a mutation at codon 12 have been detected in ~24% of human adenocarcinomas of the lung and are more prevalent in smokers than in non-smokers. The observed mutations are GGT to TGT (58%), GTT (16%) and GAT (19%) (27). The relative prevalence of G to T transversions has been cited as evidence that the mutations are due to interactions with DNA of metabolites of polynuclear aromatic hydrocarbons in cigarette smoke. However, the present results indicate that pyridyloxobutylation of DNA can also cause G to T transversions. HPB releasing adducts, formed from NNK or the related tobacco-specific nitrosamine *N'*-nitrosonornicotine, have been detected in DNA isolated from smokers' lungs, as have O^6-mG and 7-mG (8,9,28). The relative role of these adducts in activating human Ki-*ras* genes is unclear at present. While the mouse lung tumorigenesis model appears to be especially sensitive to DNA methylation particularly with respect to Ki-*ras* activation, the role of DNA methylation in the rat model is not apparent nor is it

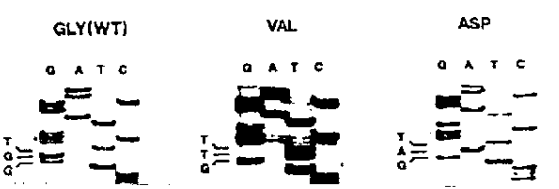

Fig. 4. Sequencing of mouse Ki-*ras* fragments. Selected samples that were found to exhibit two positive signals in our hybridization procedure were further analyzed via re-amplification, cloning, and sequencing. Shown are two different NNKOAc-induced tumor samples, one of which appeared positive for both GAT and GTT and the other for both GAT and TGT (see Figure 3). The sequence of the normal allele is shown in the left panel whereas that of GTT (not GAT) confirming a valine mutation in one tumor (middle panel) and GAT (not TGT) confirming an aspartate mutation in the other tumor (right panel).

clear whether the mouse or rat is a better model with respect to human lung cancer.

The present results therefore provide another example of DNA modifications caused by carcinogens in tobacco smoke which have the potential to result in G to T transversion mutations. Other examples include the adducts formed from polynuclear aromatic hydrocarbons, aromatic amines and α,β-unsaturated aldehydes as well as 8-oxoguanine produced as a result of oxidative damage or from 2-nitropropane (18,20,29–32). Since these DNA damaging agents are all abundant in tobacco smoke, it may be difficult to relate mutations in genes isolated from smokers to the specific carcinogens which produced them.

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